PHENOL AND CRESOL AS PRESERVATIVES IN BIOLOGIC PRODUCTS

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In the manufacture of serums, vaccines and other biologic products, it is customary to add phenol or cresol as a preservative. When added to serums, a precipitate forms, due to the interaction of the phenol or cresol with the serum proteins. Krumwiede and Banzhaf [1] developed a method of adding cresol to antitoxins which eliminated the precipitation of the serum proteins. They used equal parts of cresol and ether. The addition of this mixture to serums in a concentration of 0.4%-0.5% cresol caused little or no precipitation. The authors state that subsequent precipitation is not necessarily limited by the ether but is never greater than in cresol alone. They also claim that the mixture of ether and cresol is more strongly antiseptic than cresol alone.

Experiments were made to determine (1) the effect of cresol and ether-cresol with time on serums in relation to the amount of precipitate formed, (2) the influence of ether in the ether-cresol mixture as to (a) germicidal value, (b) hemolytic power of cresol, and (3) the mechanism of the ether-cresol action on serums. A somewhat similar study was made on the action of phenol and ether-phenol on serums.

THE ACTION OF CRESOL AND ETHER-CRESOL ON SERUMS

In order to study the comparative action of cresol and ether-cresol on serums the following experiment was performed:

(a) One hundred c c amounts of fresh horse-serum previously candle filtered were placed in nonsoluble flint bottles. To some was added cresol ranging from 0% as control to 0.6% and to others was added ether-cresol solution 1:1 in amounts from 0.2% to 1%. All the ether-cresol solution used in this experiment consisted of equal parts of ether and cresol. A series of bottles containing citrated plasma was treated in exactly the same manner as the normal serum.

It was noted that the serum and plasma receiving the straight cresol were "burned" at the point of contact between the cresol and the serum in question. This was more pronounced when the cresol was

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[1] Jour. Infect. Dis., 1921, 28, p. 367.

added at once in a continuous stream. If, however, the cresol was added drop by drop the behavior was somewhat different. The first drop spread on the surface of the serum while additional drops gradually sank to the bottom in separate "oily" globules having on the outside a coating of albuminous material. When these were shaken, solution took place but not with much "burning" action. This behavior of cresol suggested at once that surface tension and surface phenomena played a part.

The bottles treated with ether-cresol solution did not show the destructive burning action of the straight cresol; those serums receiving 0.2% to 0.6% ether-cresol solution remained clear, while the 0.8% and 1.0% bottles showed a turbidity and opalescence resembling colloidal solutions.

The normal serum and citrated plasma were kept at room temperature and observed at various intervals for precipitated protein, change of color and formation of fibrin. In order to determine the amount of precipitate somewhat quantitatively, the normal serum was centrifuged at constant high speed for 30 minutes, the supernatant siphoned off carefully, and the sediment tested for total nitrogen by the Kjeldahl method. This procedure does not give strictly quantitative results, but would show any great variations in the amount of precipitate formed.

The same method was not used for the citrated plasma because the fibrin formed in some of the bottles shortly after adding the preservative.

Tables 1 and 2 give a summary of the serums described after eight months' standing at room temperature.

Discussion of Results.—The data in tables 1 and 2 bring out these facts: 1. Cresol changes the color of serum or plasma from yellow with a reddish tinge to yellow with a definite greenish or greenish yellow tinge. 2. Although the method used for determining the amount of precipitate formed on standing is only approximately quantitative, there is no marked difference between the amount of precipitate in the serums treated with straight cresol and those treated with ether-cresol. 3. The action of cresol or ether-cresol on citrated plasma is to accelerate the formation of fibrin. Those bottles containing 0.2% or more cresol showed complete fibrin formation in a month, whereas the controls, without preservative, did not coagulate completely until after 7 months. 4. The precipitate in normal serum formed on standing was insoluble in water, cold solutions of acids and alkalis, or in salt solution. Apparently it was mostly finely divided fibrin. On shaking the precipitate went into suspension giving the serums a milky opalescence.

TABLE 1

NORMAL SERUM

Percentage of Preservative	Color	Appearance	Precipitate as Mg. Nitrogen
0 cresol	Reddish-yellow	Clear	8.5
0.1 cresol	Reddish-yellow	Clear	7.6
0.2 cresol	Greenish-yellow	Slight opalescence	9.2
0.3 cresol	Greenish-yellow	Marked opalescence	8.7
0.4 cresol	Greenish-yellow	Colloidal; muddy	9.0
0.5 cresol	Greenish-yellow	Colloidal; muddy; opaque	11.6
0.2 ether-cresol	Reddish tinge	Clear	8.2
0.3 ether-cresol	Reddish tinge	Clear	6.7
0.4 ether cresol	Greenish tinge	Clear	7.8
0.5 ether-cresol	Greenish tinge	Slight opalescence	8.5
0.6 ether-cresol	Greenish tinge	Slight opalescence	8.9
0.7 ether-cresol	Greenish tinge	Marked opalescence	9.5
0.8 ether-cresol	Greenish tinge	Colloidal; muddy	9.0
0.9 ether-cresol	Greenish tinge	Colloidal; muddy	9.8
1.0 ether-cresol	Greenish tinge	Colloidal; muddy; opaque	10.8

TABLE 2

CITRATED PLASMA

Percentage of Preservative	Color	Appearance	Fibrin
0 cresol (control)	Yellow	Coagulated	Completely formed
0.1 cresol	Yellow	Coagulated	Completely formed
0.2 cresol	Greenish-yellow	Coagulated	Completely formed
0.3 cresol	Greenish-yellow	Coagulated	Completely formed
0.4 cresol	Greenish-yellow	Turbid and coagulated	Completely formed
0.5 cresol	Greenish-yellow	Turbid and coagulated	Completely formed
0.2 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.3 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.4 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.5 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.6 ether-cresol	Greenish-yellow	Clear and coagulated	Completely formed
0.7 ether-cresol	Greenish-yellow	Turbid and coagulated	Completely formed
0.8 ether-cresol	Greenish-yellow	Turbid and coagulated	Completely formed
0.9 ether-cresol	Greenish-yellow	Turbid and coagulated	Completely formed
1.0 ether-cresol	Greenish-yellow	Turbid and coagulated	Completely formed

Another experiment was made in which a lot of normal serum was divided into 3 parts: One portion had no preservative added, a second portion had 0.3% cresol, and a third portion had 0.6% ether-cresol. The three portions were candle-filtered and placed in the refrigerator for aging. At the end of 10 months, the 3 portions were tested quantitatively for precipitate. Instead of using the previous method of determining the amount of precipitate by centrifuging and running a Kjeldahl on the sediment, the following procedure was adopted, which we think is more accurate:

One hundred c c samples of the 3 serums were centrifuged for the same length of time and same speed. The supernatant clear serum was carefully poured off, and the sediment suspended in 200 c c of distilled water. The resulting turbidity was then measured by a Jackson turbidimeter, such as is used for determining sulphates in water. The results representing the average of 3 readings and expressed as SO_3 pts. per million were as follows: (a) normal serum — no preservative, 52.0; (b) normal serum + 0.3% cresol, 63.0, and (c) normal serum + 0.6% ether-cresol, 64.0. These results are striking and show conclusively that the amount of precipitate in the ether and ether-cresol samples is the same.

THE MECHANISM OF ETHER-CRESOL SOLUTION ON PREVENTING THE BURNING OF SERUMS

As the ether-cresol solution did not "burn" the serum to the same degree as the straight cresol, it was thought important to study the reason for this behavior. Various cresol solvents were used in the proportion of 1:1. Glycerol, benzene, carbon bisulphide, chloroform, ethyl alcohol and amyl-alcohol were tried. Of these, only the amyl-alcohol behaved similarly to the ether-cresol solution, that is, it failed to "burn" the serum. A consideration of surface tension phenomenon suggested itself. Accordingly, the surface tension of normal serum without preservative was found and compared with that of normal serum containing straight cresol or ether-cresol.

In all surface tension determinations a Traube stalagmometer was used, that is, the average weight of a drop of the fluid from a standardized pipette was found. Taking the surface tension of water as unity, the surface of a solution is given by the formula:

$$\frac{\text{Specific gravity of solution} \times \text{number of drops of water}}{\text{Number of drops of solution}}$$

The result obtained is relative and not absolute; to express the tension in dynes per cm. it is necessary to multiply the ratio in the formula by 73.3, the surface tension of pure water at 18° C.

The surface tension of 6 normal serums without preservatives and with 0.3% cresol or 0.3% phenol was determined, and it was found that the cresol lowered the surface tension considerably more than the phenol. The results are given in table 3. (Surface tension of water equals 1.)

TABLE 3
THE EFFECT OF CRESOL ON THE SURFACE TENSION OF SERUM

Serum No.	Without Preservative	With 0.3% Cresol (Redistilled)	With 0.3% Phenol (Redistilled)
91,563	0.94	0.70	0.82
92,183	0.86	0.64	0.76
91,693	0.83	0.63	0.74
92,399	0.90	0.66	0.78
3,932	0.83	0.64	0.73
4,953	0.92	0.67	0.79

Table 3 shows that cresol lowers the surface tension markedly, an average of about 25%, whereas, phenol lowers it only about one-half that amount.

Lowering of surface tension and concentration was then studied. It was found that small amounts of cresol lowered the tension a certain amount while each additional quantity lowered it less and less.

TABLE 4
THE EFFECT OF CRESOL ON THE SURFACE TENSION OF SERUM

Percentage of Cresol	Tension
0 (no preservative)	0.86
0.1	0.80
0.2	0.70
0.3	0.67
0.4	0.65
0.5	0.64
0.6	0.62

The surface tension of serum containing 0.6% ether-cresol was found to be more or less the additive effect of cresol and ether, thus

TABLE 5
THE EFFECT OF ETHER-CRESOL ON THE SURFACE TENSION OF SERUM

Serum	Tension
No preservative	0.85
0.3% cresol	0.64
0.3% ether	0.74
0.6% ether-cresol	0.58

Before discussing the significance of the lowering of surface tension by cresol and phenol it is well to attempt to explain the reason why

straight cresol "burns" the serum while the ether-cresol does not. According to Gibb's theorem, a substance which lowers the surface tension of a solvent becomes more concentrated in the surface film than in the interior. We are dealing with a system of two phases, serum and ether. For simplicity's sake we may assume that ether is immiscible in serum, then the cresol which lowers the surface tension of the serum concentrates in the surface layer next to the ether. As cresol is much more soluble in ether than in serum and as it does not lower the surface tension of ether, it will diffuse into the layer of ether until an equilibrium is established in the surface layer of the serum. Expressed in general terms a fluid 3 spreads over the common boundaries of two fluids, 1 and 2, whenever

$$\sigma\,{}^{1}\!/_{2} > \sigma\,{}^{2}\!/_{3} - \sigma\,{}^{3}\!/_{1}$$

where $\sigma 1/2$ = surface tension of serum against ether
$\sigma 2/3$ = surface tension of ether against cresol
$\sigma 3/1$ = surface tension of cresol against serum

Therefore, when the ether-cresol is added to the serum the cresol concentrates in the layer of ether. However, by constant shaking and on standing an equilibrium is established between the cresol in the ether and that in the serum. Ether itself is soluble to some extent in the serum, so that the final effect of the ether on the cresol is to distribute the latter more intimately in the serum. Hence, we have the action of the cresol on the serum evenly distributed with a resulting colloidal opalescence, instead of the selective "burning" of the straight cresol which acts in a concentrated form on that part of the serum with which it comes in contact.

PHENOL COEFFICIENT OF CRESOL AND ETHER-CRESOL SOLUTION

Banzhaf and Krumwiede [1] state that ether-cresol is more strongly antiseptic than cresol alone. We have not been able to confirm their results. The phenol coefficient of an ether cresol solution 1:1 and straight cresol was determined according to the U. S. Hygienic Laboratory method, using distilled water for diluting. The results are shown in table 6. The experimental data shows that ether-cresol is not more strongly germicidal but slightly less germicidal than cresol alone. It is true that ether has antiseptic properties but that depends on the concentration used. If the concentration is high, the ether exerts its germicidal action on bacteria by its power to penetrate their cell membrane. A concentration of 0.3%, the amount used in serums, is not strong enough to exert any germicidal action.

If we take a suspension of bacteria and regard it as a dispersed phase, the suspension must obey certain physical laws. A suspension of organisms has large surface development, and therefore it will adsorb with great affinity those substances which lower the surface tension of water. The amount adsorbed is greater, the more the dissolved substance lowers the surface tension. Therefore, cresol should be adsorbed much more than phenol. When ether cresol is added to a bacterial suspension, the adsorption equilibrium is disturbed. The cresol is less adsorbed by the bacteria due to the fact that the ether increases the surface tension between the bacterial suspension and cresol. As a result the cresol is less adsorbed and its antiseptic power diminished. Paul and Krönig [2] found this to be true with

TABLE 6

HYGIENIC LABORATORY TEST WITH B. TYPHOSUS

Substance	Dilution	Time in Minutes						Date
		2½	5	7½	10	12½	15	
Cresol	250	—	—	—	—	—	—	1/12/21
	300	—	—	—	—	—	—	
	350	—	—	—	—	—	—	
	400	—	—	—	—	—	—	
	450	+	—	—	—	—	—	
Cresol-ether	250	—	—	—	—	—	—	1/12/21
	300	—	—	—	—	—	—	
	350	—	—	—	—	—	—	
	400	+	+	—	—	—	—	
	450	+	+	+	—	—	—	
Cresol	300	—	—	—	—	—	—	6/7/21
	350	—	—	—	—	—	—	
	400	+	+	—	—	—	—	
	450	+	+	—	—	—	—	
	500	+	+	+	+	+	+	
Cresol-ether	300	—	—	—	—	—	—	6/7/21
	350	—	—	—	—	—	—	
	400	+	+	+	—	—	—	
	450	+	+	+	+	—	—	
	500	+	+	+	+	+	—	

anthrax. Their results showed that phenol was far more germicidal on the spores when used as an aqueous solution than as an alcoholic solution.

SURFACE TENSION AND HEMOLYSIS

Woodward and Alsberg [3] showed that the lowering of surface tension by saponins and their hemolytic power did not run parallel. As phenol and cresol are known to hemolyze blood corpuscles, it was decided to study the comparative hemolytic power of phenol and cresol and the relation to the lowering of surface tension.

[2] Colloids in Biology and Medicine, p. 395.

[3] Jr. Pharm. and Expt. Therap., 1920, 16, p. 237.

The cytolytic power of disinfectants varies with their penetrating power, and should vary also with their power to lower surface tension. Therefore, cresol should be more effective than phenol.

Experiments showed this to be true. The technic employed for determining hemolysis was that of Woodward and Alsberg.[3] Different percentages of phenol or cresol in Locke solution were made. Ten c c of the solution at 37 C. were placed in test tubes, and then 2 drops of washed sheep erythrocytes were added to each. The tubes were mixed by inverting and then placed in the incubator at 37 C. The relative hemolytic activity was noted every 10 minutes, for one hour. With higher concentration of cresol hemolysis took place within less than 5 minutes.

The surface tension of each solution was determined at 18 C. although the hemolysis took place at 37 C. This introduces an error but as we are interested in comparative results, the error plays no part in the final conclusion. The results obtained are given in table 8 for cresol, ether-cresol, phenol and ether-phenol. The tubes containing the ether were stoppered to prevent evaporation.

TABLE 7

RESULTS OF EXPERIMENTS

Substance	Tension	10 Min.	20 Min.	30 Min.	40 Min.	50 Min.	60 Min.
Water	1.00						
Locke solution	1.00	0	0	0	0	0	0
Locke solution + 0.1% cresol	0.91	0	0	0	0	0	0
Locke solution + 0.2% cresol	0.80	0	0	+	+++	+++	+++
Locke solution + 0.3% cresol	0.74	++	+++	+++	C	C	C
Locke solution + 0.4% cresol	0.70	+++	+++	C	C	C	C
Locke solution + 0.5% cresol	0.65	+++	+++	C	C	C	C
Locke solution + 0.3% ether	0.90	0	0	0	0	0	0
Locke solution + 0.2% ether-cresol	0.80	0	0	0	0	0	0
Locke solution + 0.4% ether-cresol	0.75	0	0	0	0	++	+++
Locke solution + 0.6% ether-cresol	0.70	+	++	+++	+++	C	C
Locke solution + 0.8% ether-cresol	0.65	+++	+++	C	C	C	C
Locke solution + 1.0% ether-cresol	0.60	+++	C	C	C	C	C

0 = no lysis; + = slight lysis; ++ = considerable lysis; +++ = complete lysis; C = complete lysis with coagulation of protein.

* The composition of the Locke solution was as follows: NaCl, 9.0 gm. per liter; KCl, 0.42 gm. per liter; $CaCl_2$, 0.24 gm. per liter; $NaHCO_3$, 0.10 gm. per liter.

A study of table 7 brings out that (1) cresol produces hemolysis of the erythrocytes much more rapidly than phenol; (2) the rate of lysis is directly proportional to the concentration of the cresol or phenol and to the lowering of the surface tension; (3) ether-cresol produces hemolysis just as readily as cresol; (4) the same relation is true for phenol and ether-phenol; (5) although ether lowers the surface tension, it in itself does not produce hemolysis.

Discussion of Results.—The fact that ether itself lowers the surface tension without producing hemolysis is significant. Merely lowering the surface tension does not necessarily mean hemolysis, but on the other hand substances which lower the surface tension are easily

adsorbed by the erythrocytes. Cresol, therefore, produces hemolysis more rapidly than phenol because it is adsorbed more than phenol and is thus more effective in bringing about cytolysis.

Another important difference in the behavior of the two substances is that cresol acts on the erythrocytes in two distinct ways: (1) it causes cytolysis with production of hemolysis, and (2) it acts on the liberated hemoglobin and coagulates the protein faction or globin. Phenol in a concentration of 0.5% produces hemolysis in one hour but does not destroy the globin. This means that cresol is far more active on proteins than phenol.

Summary.—A study of preservatives including cresol, ether-cresol, phenol and ether-phenol on serum and plasma brings out that: (1) cresol or ether-cresol changes the color of serum or plasma from a light yellow to a greenish yellow; (2) there is no marked difference in the amount of precipitate formed on standing between serums treated with straight cresol or ether-cresol; (3) the precipitate formed in normal serum is mostly finely divided fibrin; (4) cresol hastens the formation of fibrin in plasma; (5) cresol lowers the surface tension of serum much more markedly than phenol; (6) ether-cresol does not "burn" serum on account of a surface tension phenomenon; (7) cresol produces hemolysis rapidly with destruction of the hemoglobin while phenol produces only slight hemolysis with no effect on the hemoglobin under the conditions of the experiment; (8) ether does not alter the course of hemolysis in itself or as ether-cresol or ether-phenol; (9) the lowering of surface tension in itself does not produce hemolysis, but substances which lower the surface tension are adsorbed by the erythrocytes to a greater degree.

CONCLUSION

Experimental data do not show that ether-cresol has any advantages as preservative of serum over straight ether in the total precipitate formed on standing or in germicidal value.